CHAPTER-V

EVALUATION OF HORMONE-LIKE ACTIVITIES OF RICE PEPTIDES RELATED WITH GERMINATION AND GROWTH

5.1 INTRODUCTION

Rice is the important staple food for more than half of the world population and occupies 11 percent of world agricultural land. The plant rice belongs to the genus *Oryza* of family Poaceae. The genus *Oryza* has 24 species, of which only two species are cultivated namely *O. sativa* and *O. glaberrima* and the rest 22 species are wild. The varieties of *O. sativa* are mainly cultivated in Asia, America and Europe whereas the varieties found in West Africa belong to species *O. glaberrima*. Further, *O. sativa* rice varieties of the world are commonly clustered into three sub-species -viz. indica, japonica and javanica. The varieties developed in Japan and Indonesia is recognized as japonica and javanica respectively while the rice grown in India belongs to indica.

India is the second largest producer of rice in the world after China and occupies about 23.3 percent of gross cropped area of the country. Rice contributes 43 per cent of total food grain production and 46 per cent of total cereal production. Just after independence i.e. during 1950-51, area under rice cultivation was 30 million hectares (Mha) and its production at that time was only 35 million tonnes. In 2008, this figure has increased to 44 mha but the production has raised a significant dimension of 99 million tonnes (MT). Therefore the country witnessed an impressive growth in rice production in the post-independence era due to the adoption of semi dwarf high yielding varieties coupled with the adoption of intensive input based management practices. Rice production was increased four times, productivity three times while the area increase was only one and half times during this period. In order to keep pace with the growing population, the estimated rice requirement by 2025 is about 130 MT. But the saturating trend in the yield of high yielding varieties, declining and degrading natural resources like land and water and acute shortage of labour make the task of increasing rice production quite challenging in recent decades. Actually the phenomenal swift in increase in rice production and productivity has been uneven, and the disparity is highly pervasive among the states and across the diverse ecosystems. Moreover, the yield curve has started showing slight declining trend in the later half of the nineties and have been continuing thereafter, which appears that the agro techniques related with the cultivation of rice is not sustainable. The gain due to modern rice technology has been discriminatory against

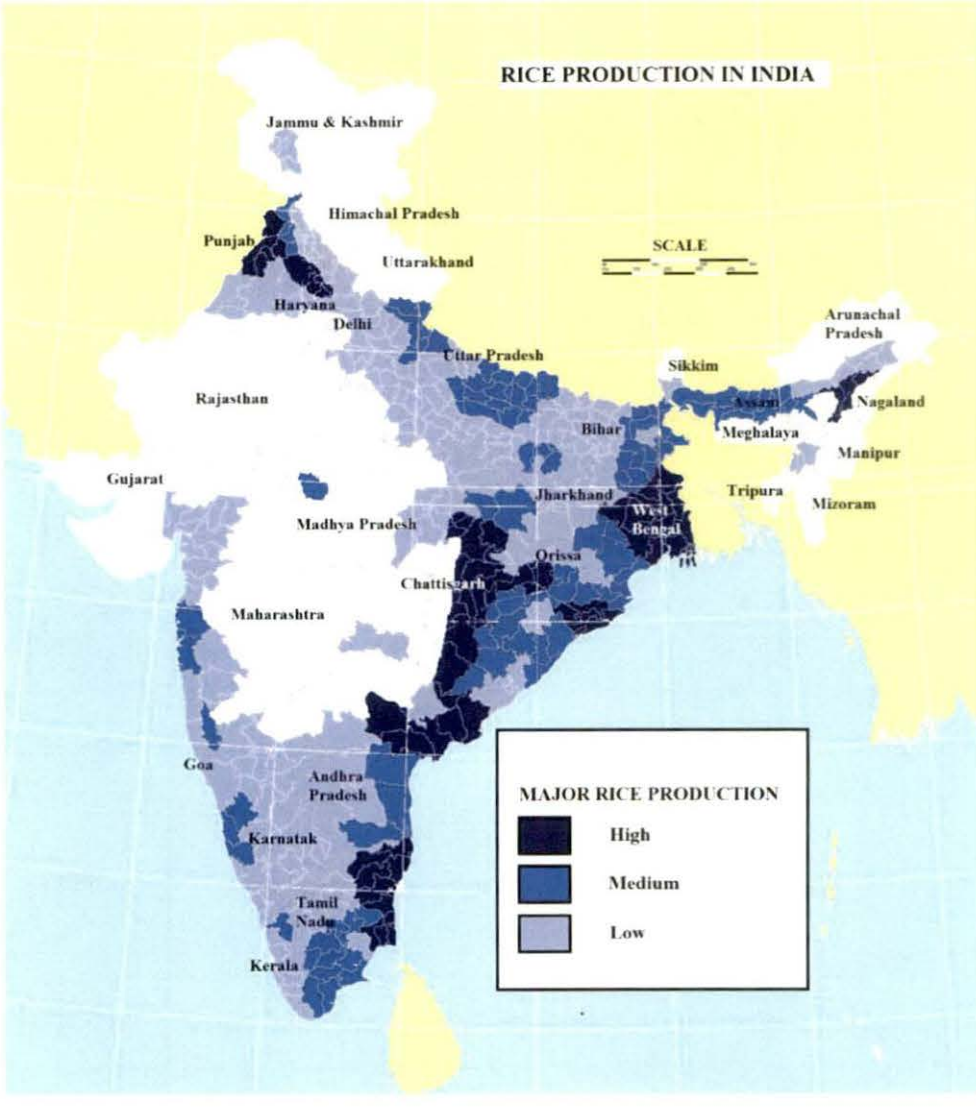


Figure 5.1 Annual production of rice in India

the resource poor areas, mostly dominated by small and marginal farmers. Productivity ranges from a less than 2 tonnes/ha in rain fed areas to as high as 5.85 tonnes/ha in irrigated tract in Punjab (Figure 5.1). This disparity is caused as the research achievement failed to fulfil the requirements of demand-driven technology to the target groups for wider adoption.

In West Bengal, the area and production under food grains during 2007-2008 were 6.37 Mha and 16.06 MT respectively, out of which rice cultivation shares 91% in area and 93% in production. Rice grows in this state in three different seasons which are Aus (autumn rice), Amon (winter rice) and Boro (summer rice). The percentage shares of these three categories of rice were 4.92, 68.65 and 26.43 with respect to area and similarly 3.84, 62.69 and 3.47 in production of total rice respectively. In West Bengal production of rice has attained a continuous upward trend till 2007-2008 and increased substantially in a similar fashion, while the area under rice cultivation has increased slightly. All these changes in productivity are mainly due to introduction of improved high yielding varieties and enhancement of irrigation facilities along with improved package of practices followed by the farmers of West Bengal.

Flood-prone lowlands are the major areas for extensive cultivation of rice in most of the countries. One of the major problems for rice production in this area is poor germination and seedling establishment with direct seeding methods due to unpredictable flooding events (El-Hendawy *et al.*, 2011). Although direct seeding is advantageous over transplanting in terms of labour requirement (Tuong *et al.*, 2000), many aroma-based prime quality and high yielding rice cultivars are not good performers in direct seeding, resulting in slow development of direct seeding technology in flood-prone areas. Generally rice plants are known to be adopted to flood condition but during germination period many cultivars are sensitive to low oxygen levels due to flooding (Yamauchi *et al.*, 1994). Because low oxygen levels enhance coleoptiles elongation during the germination of rice seeds (Perata *et al.*, 1986; Redona and Mackill, 1996), several investigators have taken this as evidence and tried to relate coleoptile elongation that are initiated by water uptake with tolerance to anaerobic conditions. According to Black (1994), water uptake by the seeds follows a triphasic pattern: Phase-I represents rapid water uptake followed by a plateau phase (Phase-II) and then a post-germination phase of

water uptake (Phase-III) as discussed details in earlier chapters. Many reports have shown that the activities of biochemical changes in seeds during germination which are essential for radical protrusion, are influenced with water uptake pattern during three phases and are affected by many factors like seed structure, physiological potential, germination conditions and genotype (Mandal *et al.*, 2008; Cho, 2010). Yang *et al.* (2007) considered the relationship between water uptake patterns and biochemical changes during seed germination and reported that the protease mediated degradation of storage proteins mainly happened in the late stage of germination phase II (imbibed for 48 hours) while that of seed maturation and desiccation proteins occurred at the early stage of phase II (24 hours imbibition) when seeds were imbibed at ambient temperature. El-Hendawy *et al.*, (2011) concluded that rapid water uptake during first two days of imbibition of rice probably plays a key role in the ability to germinate under submerged condition. Similar observation was also found by Olisa *et al.*, (2010) who reported that rapid imbibition was a cause of reduced germination time for pigeon pea. This could be explained by the fact that rapid water uptake during second germination phase may lead to quick activation of α-amylase which plays a significant role in the degradation of starch into soluble sugars as the main substrate necessary for generating the energy required for growth and maintenance processes (El-Hendawy *et al.*, 2011). In addition, rapid water uptake under anaerobic conditions may lead to rapid sugar mobilization between the endosperm and embryo. Ismail *et al.* (2009) stated that the ability of tolerant genotypes of rice to degrade starch into soluble sugars under energy minimized condition probably explains their ability to grow faster under anaerobic stress. So for getting enhanced rate of germination and healthy seedling vigour anoxia tolerant genotypes are required which can able to generate and mobilize soluble sugars more rapidly by imbibing water in enhanced rate. Indeed the report of Huang *et al.* (2003) reflects the same phenomena where anoxia-intolerant cultivar IR-22 grew much slower than anoxia-tolerant type Amaroo.

But anoxia-tolerant type does not always execute better traits in terms of quality and yield. Several locally available aroma rice varieties can't tolerate anoxic or hypoxic condition in an efficient way. For converting these susceptible rice cultivars into better acclimated form against anoxic stress, there is still much to learn about the biochemical

and molecular basis of anaerobic rice germination. Tolerance to low oxygen availability is likely to be due to interaction of several physiological factors. Under anoxia, the rice coleoptiles elongates, reaching a length greater than that of the aerobic one. Tolerance of the rice coleoptiles may be due to shift from cell division to cell expansion, a process which is less energy-requiring than protein synthesis (Atwell *et al.*, 1982). Though the activity of expansin and apoplastic acidification is required for cell wall expansion, this is not operated through auxin (Pegoraro *et al.*, 1988). In anoxic tissue, apoplastic acidification could be due to putrescine mediated H^+-ATPase activation on the plasma membrane (Reggiani *et al.*, 1992); which subsequently up-regulates the transcript of expansin EXPA 7 and EXPB 12 in rice coleoptiles (Lasanthi-Kudahettige *et al.*, 2007). By this way polyamines play an important role during germination of rice seedlings. Production of α-amylase during germination also plays an important role for tolerance of anoxia in rice. Under aerobic conditions α-amylase production is enhanced in response to gibberellins produced by embryos; but the recent results indicate that gibberellins are not required for the anaerobic germination of rice grains (Loreti *et al.*, 2003).

Rice, with its many varieties and wide adaptability is the only crop that can be successfully germinated and grown in different water logged areas of tropical Asia. The matters discussed in previous paragraph conclusively state that the classical hormones can rarely influence the germination of cereals under anoxia or hypoxia, and the phenomena is more suitable for submerged rice cultivation. Therefore other molecules like polyamines or peptides besides five classical hormones are definitely involved in controlling the germination, water homeostasis and senescence physiology of rice grown in submerged condition. Also wide level of fluctuations of endogenous plant hormones in isolated rice embryos during embryogenesis and early stage of germination indicates the influence of other signal molecules during these processes. Conversely, during aerobic germination of rice, it was observed that the balance between GA_1 and ABA, rather than their absolute contents, controls the process throughout the development (Jun *et al.*, 2003). So even during aerobic condition, some other signalling system probably regulates the ration of these hormones, till now which is not very clear. In an effort to sustain rice as a reliable food crop, design and establishment of scientific research to ameliorate agronomically important traits like grain size, vivipary, uniform germination and seedling

vigour have become a hot focus in recent years (Martinez-Andujar *et al.*, 2012; Nambara *et al.*, 2010). Recent studies indicate that rice grains possess a mechanism that enables them to survive for longer period of time in dormant condition before germination and successful seedling establishment (Costa *et al.*, 2012). The findings of Sano *et al.*, (2012) indicate that while mature grains contained stored mRNAs sufficient to initiate germination events, *de novo* translation was essential for successful completion of rice seed germination. Furthermore recent investigations provide novel insight into how plants have the ability to secrete discrete long-lived mRNAs to be translated during early germination while others are targeted for degradation. So scientific investigations only on five classical hormones (auxin, gibberellins, cytokinin, ethylene and abscisic acid) doesn't enlighten enough on developing quality traits of rice as their role is seemingly limited in regulating huge transcript pattern of rice during germination. Recent studies have indicated that small plant peptides can able to regulate all aspects of growth and development by controlling the pattern of transcription as well as translation. Small peptides possessing a protein dimerization motif form non-functional heterodimers with a group of specific transcription factors and inhibiting their transcriptional activation as revealed in *Arabidopsis* (Yun-Ying *et al.*, 2008). Similarly Ubiquitin derived small peptides may serve for selective protein degradation followed by post-translational regulation and these peptides can now be considered as prime players in plant cell regulation (Downes and Vierstra, 2005). To date, post-translationally processed small hormonally active peptides have been shown to have diverse function in pants, including nearly every aspects of development and morphogenesis. But the actual role of peptides in plants is less well defined. The first unambiguous demonstration of a pool of small peptides in plant tissues described the presence of millimolar concentrations of small peptides in the endosperm of germinating cereal grains (Higgins and Payne, 1981). Preliminary data indicated that peptides only serve as a supply of nutrients to support the growth of cereal embryo. Subsequently, it has become apparent that small peptides also play a role in control of plant cell differentiation and organogenesis in addition to a nutritional role (Yang *et al.*, 2000). In rice, plant growth promoting culture solution was developed to enhance the accumulation of peptides which are pharmacologically active and having antimicrobial, antihypertensive, cholesterol-lowering, antithrombic and

antioxidant activities (Wen *et al.*, 2009). Also rice oligopeptide transporters (OsOPTs), expressed during germination were identified, their expression profile and function were well characterized (Ouyang *et al.*, 2010). Oligopeptide transporters transport a range of different types of peptides and their derivatives across membranes in an energy dependent manner (Lubkowitz *et al.*, 1998). Some of OsOPTs play important roles in the mobilization of organic nitrogenous compounds and usually associated with tissues that show signs of rapid protein turnover, such as germinating seeds and senescent leaves (Liu *et al.*, 2012).

But till date, to our knowledge no scientific reports are available related with hormone action and other physiological roles of rice peptides. So, in this chapter the study was undertaken to investigate the physiological roles related with hormone mimicking action of peptides isolated from rice seedlings. Attempts were also made to compare the role of peptides with standard hormones and their possible interaction. Finally the bioactive peptides were partially purified through gel exclusion chromatographic method and HPLC for determining the bioactivity of separated peptides.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials used for isolation of peptides

Monocotyledonous plant: Seven days old seedlings of *Oryza sativa* L. (cv. IR-22) [Rice], of family Poaceae, was taken for peptide isolation and purification.

5.2.2 Plant Materials used for Bioassay

A] *Triticum aestivum* L. (cv. Sonalika RR-21) [Wheat], or *Hordeum vulgare* L. (cv. Br. 32) dwarf variety [Barley seeds], Poaceae embryoless half seeds were taken for amylase induction assay.

B] *Colocasia esculenta* (L.) Schott, Araceae or *Commelina benghalensis* L., Commelinaceae lower epidermal peelings having stomatal guard cells were used for measuring opening and closing of stomata and aperture regulation.

C] *Raphanus sativus* L. [Radish], leaf discs were used for chlorophyll retention.

D] *Oryza sativa* L. [Rice], for coleoptile elongation.

5.2.3 Seed germination and culture condition

For isolation of peptides, rice seeds were germinated and maintained with culture conditions as specified by Wang *et al.*, 2010 with some modifications. Seeds of rice were primarily washed with running water for removal of debris, and were soaked in 1% (w/v) sodium hypochlorite solution for 15 min and then rinsed three times with sterile distilled water. Fifty rice seeds were placed in a Petri dish of 9 cm diameter with two sheets of filter paper, to which 10 ml of distilled water was added. The solution was replaced every 48h to maintain distilled water volume. All Petri dishes were placed in seed germinator (REMI made) at (25 ± 1)° C for 7 days with a 12-h light / 12-h dark photoperiod. After one week, rice seedlings with endosperm were again surface sterilized with 80% ethanol for 5 min, washed thoroughly with sterile water, and placed under laminar air flow for removal of traces of water after blot drying.

5.2.4 Isolation and purification of peptides

Cryocrushing, cold centrifugation, cation and anion exchange resin separation, ether fractionation, ultrafiltration and Sephadex LH-20 based purification of isolated peptides were performed according to the methods discussed in details in Chapter III Section 3.2.2.

5.2.5 Bioassays mimicking hormone action

5.2.5a *α-Amylase Induction/Repression*:

This bioassay was performed by incubating the embryoless half of wheat or barley seeds for 48h and amount of α-Amylase induction/repression test was verified by the presence of reducing sugar through 3,5 Dinitrosalicylic Acid (DNSA) method (Chrispeels and Verner, 1967).

5.2.5b *Stomatal Guard Cell opening/closing:*

Stomatal epidermal peels were kept for 3h to 5h in light/dark and their opening/closing was measured in microscope pre-calibrated with stage and ocular micrometer. These peels were then transferred to peptide solution of different

concentrations or untreated control and be kept in light/dark to observe the initiation of closing/opening (Smith and Willmer, 1981).

5.2.5c *Chlorophyll Retention Test:*

Green leaf discs of radish *Raphanus sativus* L. was aseptically applied to different concentration of peptides against control and after four days, chlorophyll content was measured (Lichtenthale and Wellburn 1983; Arnon *et al.*, 1949).

5.2.5d *Coleoptiles' Elongation Assay*:

The assay was performed as prescribed by Meidner (1984) and specified in details in Chapter IV Section 4.2.9.

5.2.6 HPLC analysis of bioactive peptide fractions

Mentioned briefly in Chapter III Section 3.2.13

5.3 RESULTS AND DISCUSSIONS

5.3.1 Induction of amylases during germination

The seedling development of cereal grains may be divided into three well defined stages; imbibition, germination and elongation of seedlings (seedling vigour) (Thomas and Rodriguez, 1994). After imbibition of seeds, sugars are rapidly consumed by the embryo leading to starvation of sugar and subsequently induction of α-amylase gene takes place during germination (Yu *et al.*, 1996). The phytohormone gibberellins (GA) are released from the embryo and stimulates aleurone cell layer surrounding the starchy endosperm from which the secretion and synthesis of α-amylase and other hydrolases take place (Chen *et al.*, 2006). GA is synthesised exclusively from scutellar epithelium of monocot embryo and transcriptional activation of hydrolytic enzymes in aleurone layers is achieved by this phytohormone (Kaneko *et al.*, 2002). As the induction of α-amylase is sensitive to exogenous GA application, amylase secretion has been most extensively studied since it plays a unique role in the degradation of starch (Mitsunaga *et al.*, 1994).

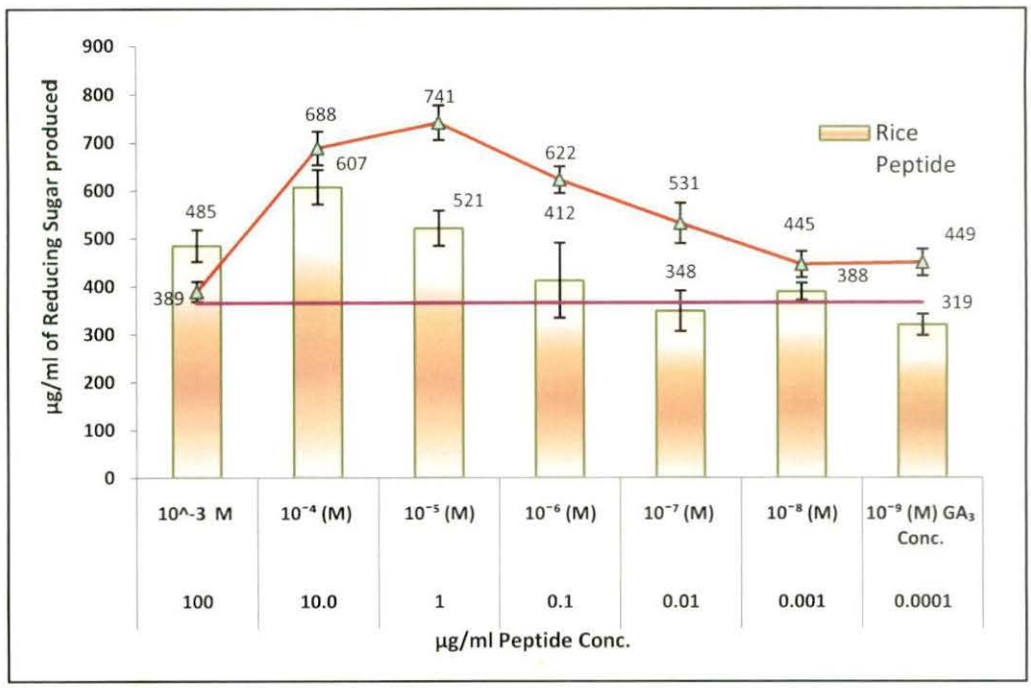


Figure 5.2 Comparative study of α-amylase induction of barley seeds by GA_3 and semi-pure peptides (3000-500 Da) of rice

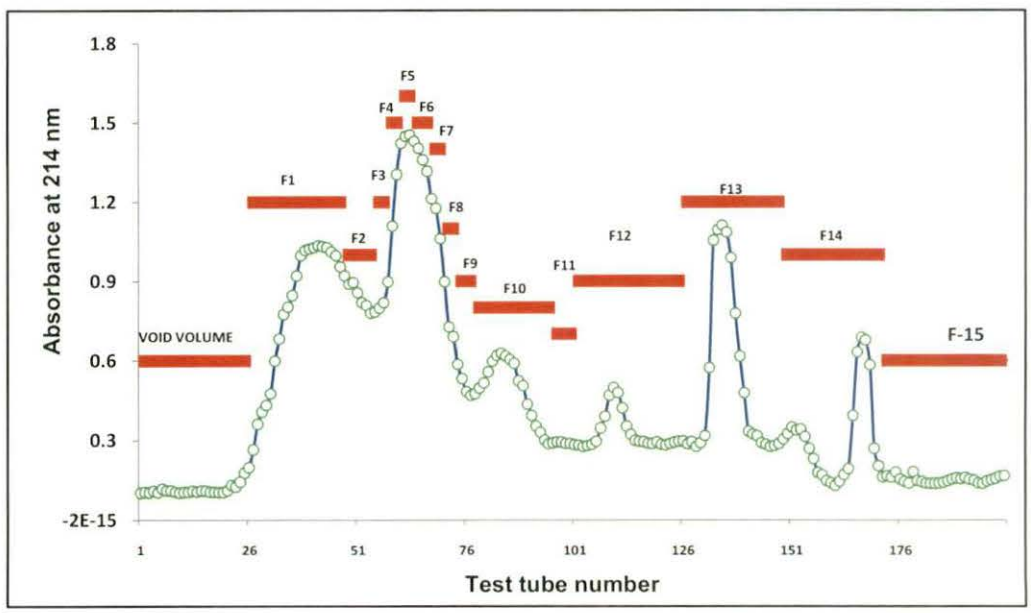


Figure 5.3 Absorbance profile of peptide fractions distributed in different test tubes after Sephadex LH-20 purification

In this study, for analysis of induction or repression of α-amylases by isolated peptides, GA was taken as standard, as this phytohormone is frequently used in various biochemical experiments associated with signalling of different hydrolases. Amylase induction on embryo-less half barley seeds by GA_3 was gradually enhanced with the increasing applied hormone concentrations and the optimum response was achieved with 10^{-5} (M) GA_3 concentration, where 741 μg/ml of reducing sugar was produced (Figure 5.2). Besides gibberellins, other different components unrelated with phytohormones may contribute in inducing amylases from aleurone layers. Previous studies indicated that pre-treatment of rice aleurone cells with sulphuric acid induced amylase activity but interestingly amylase activation on wheat aleurone cells was not found with similar treatments (Mitsunaga *et al*, 2007). Sterilizing agent sodium hypochlorite has also been reported to induce the synthesis and secretion of α-amylases in the cotyledons of *Vigna radiata*, even when detached from embryo axis (Kaneko and Morohashi, 2003). Mastoparan-7, a cationic amphiphilic toxic tetradecapeptide, originally isolated from wasp venom can also induce α-amylase through activation of heterotrimeric G-proteins, as established in wild oat aleurone protoplast culture cells (Jones *et al.*, 1998). In rice seeds, a versatile group of oligopeptides are available after enzymatic hydrolysis (Wang *et al.*, 2013), some of which are pharmacologically very active (Shih and Daigle, 2000). Unfortunately nothing has been accounted on the bioactivity of oligopeptides synthesised during germination of rice seedlings. In this investigation, the dose-dependent induction or inhibition of amylases of germination induced peptides isolated from rice seedlings were evaluated in embryoless-half barley seeds, as it is possible to discard the effects of embryo derived gibberellins through this process (Mitsunaga *et al.*, 2007). Maximum amount of reducing sugars were produced (607 μg/ml) by incubating embryoless-half barley seeds in 10 μg/ml peptide solution for 48 hours (Figure 5.2). Though the induction of amylases by isolated rice peptides didn't reach the response produced by optimal GA doses, the maximum activity obtained through peptide treatment was significantly higher (56.07%) than control (Figure 5.2). A gradual decline in amylase induction was observed with peptide treatment when the applied doses were reduced from 10μg/ml to 10ng/ml (Figure 5.2).

Table 5.1 Amylase induction in embryoless half barley seeds by incubation with three specified concentrations of LH-20 purified fractions of semi-pure rice peptides (500-3000 Da)

Peptide Fraction No.	Amylase activity in different concentrations of peptides [µg/ml of Reducing Sugar produced (Mean ± SD), with three replicates]					Amylase induction with peptides [Mean percentage increase/decrease over control]			
	Control	Peptide concentrations				Peptide concentrations			
		100 ppm	10 ppm	1 ppm	0.1 ppm	100 ppm	10 ppm	1 ppm	0.1 ppm
F1	402 ± 25	392 ± 26	388 ± 19	354 ± 25	344 ± 24	-2.49	-3.48	-11.94	-14.43
F2	395 ± 33	391 ± 25	412 ± 24	416 ± 22	404 ± 22	-1.01	4.30	5.32	2.28
F3	405 ± 21	416 ± 36	456 ± 28	485 ± 26	444 ± 25	2.72	12.59	19.75	9.63
F4	402 ± 27	432 ± 25	546 ± 25	522 ± 35	479 ± 32	7.46	**35.82**	**29.85**	**19.15**
F5	382 ± 22	495 ± 26	644 ± 34	581 ± 26	538 ± 22	**29.58**	**68.59**	**52.09**	**40.84**
F6	385 ± 18	543 ± 26	502 ± 18	488 ± 28	426 ± 28	**41.04**	**30.39**	**26.75**	**10.65**
F7	388 ± 18	422 ± 33	468 ± 25	401 ± 24	372 ± 28	8.76	20.62	3.35	-4.12
F8	415 ± 28	376 ± 26	422 ± 28	378 ± 34	368 ± 26	-9.40	1.69	-8.92	-11.33
F9	411 ± 26	428 ± 18	415 ± 24	404 ± 24	388 ± 21	4.14	0.97	-1.70	-5.60
F10	408 ± 24	401 ± 22	385 ± 20	381 ± 32	379 ± 24	-1.72	-5.64	-6.62	-7.11
F11	418 ± 25	406 ± 24	382 ± 16	378 ± 26	369 ± 24	-2.87	-8.61	-9.57	-11.72
F12	385 ± 24	392 ± 34	354 ± 22	366 ± 28	378 ± 18	1.82	-8.05	-4.94	-1.82
F13	395 ± 32	386 ± 30	368 ± 29	376 ± 24	385 ± 24	-2.28	-6.84	-4.81	-2.53
F14	408 ± 31	395 ± 24	372 ± 34	368 ± 25	373 ± 26	-3.19	-8.82	-9.80	-8.58
F15	391 ± 28	390 ± 34	388 ± 26	382 ± 24	361 ± 23	-0.26	-0.77	-2.30	-7.67

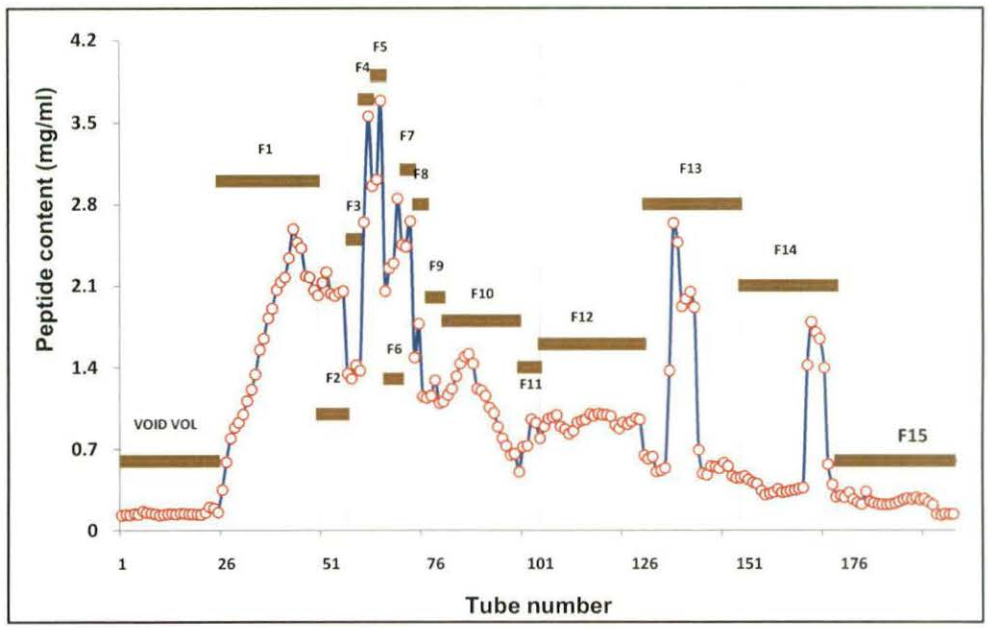


Figure 5.4 Profile of amount of peptides distributed in different test tubes after Sephadex LH-20 purification

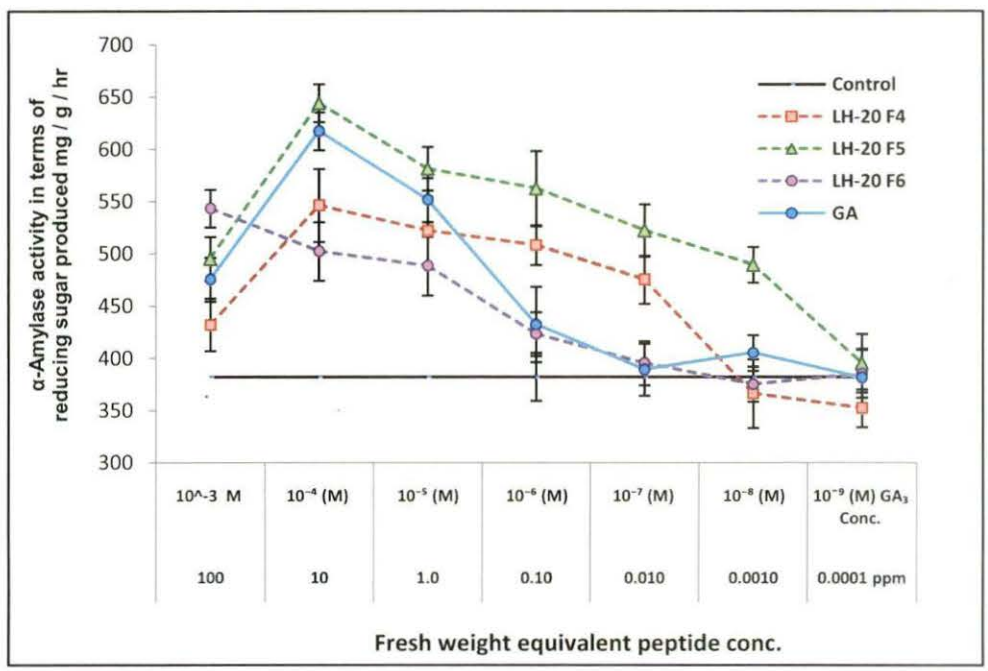


Figure 5.5 Comparative study of amylase inducing activity with GA_3 and different concentrations of LH-20 separated fractions of rice peptides- F_4, F_5 and F_6 with control

For identification of specific peptides responsible for amylase induction, purification through Sephadex LH-20 was performed after ultrafiltration of heterogeneous rice peptides. Peptides eluted from Sephadex gel column were collected in 200 different test tubes with 5 ml each through automated fraction collector associated with peristaltic pump. Out of 200 test tubes collected, first 26 tubes (130 ml) were considered as void volume of column and rejected. Peptides were distributed in the remaining 174 test tubes, which were clustered into fifteen different fractions in accordance with UV absorption profile represented in Figure 5.3. UV absorption was high at fractions F_1, F_3 to F_7, F_{13} and F_{14} (Figure 5.3). A prominent peak was observed between F_3 to F_9, which culminates at F_5 (Figure 5.3). The ninhydrin based peptide profile of LH-20 is represented in Figure 5.4, in which sharp peaks are observed in F_4, F_5, F_{13} and F_{14}, indicating that peptides were concentrated in those fractions. Amylase inducing properties of these peptide fractions were determined with four specified concentrations (100 to 0.1 ppm) in embryoless half barley seeds. Among these, best response was obtained from fraction F_4, F_5 and F_6, and highest level of amylase activity was observed after incubation with 10 ppm F_5 peptide fraction, where 68.59% increase of amylase induction was realized from control (Table 5.1). Besides this concentration, other different doses of F_5 were also essentially bioactive when amylase induction was considered. Amylase activity in terms of better yield of reducing sugar per unit grains was also noted after 48 hrs incubation of barley seeds with 100 and 1 ppm F_4 applied peptides doses (Table 5.1). Peptide fraction F_6 exhibited dose-dependent response of amylase induction in between 100 to 0.1 ppm concentration, i.e. the response was relapsed gradually with the dilution of peptide treatment (with maximum of 41% and the minimum of 11% induction was noticed over control within the supplied range of peptide doses) (Table 5.1). More intensive analysis of amylase induction was performed with bioactive peptide fraction F_4, F_5 and F_6 by taking wide range of peptide doses from 100 to 0.0001 ppm (Figure 5.5). Here also maximum amylase activity was documented with F_5 peptide application where optimum activity was restricted at 10 ppm applied dose (Figure 5.5). The activity was even better or comparable with standard phytohormone GA_3 and was effective up to 1 ng/ml of applied peptide doses, which indicates the high sensitivity of this peptide fraction towards aleurone layers (Figure 5.5). As stated in Table 5.1, F_6

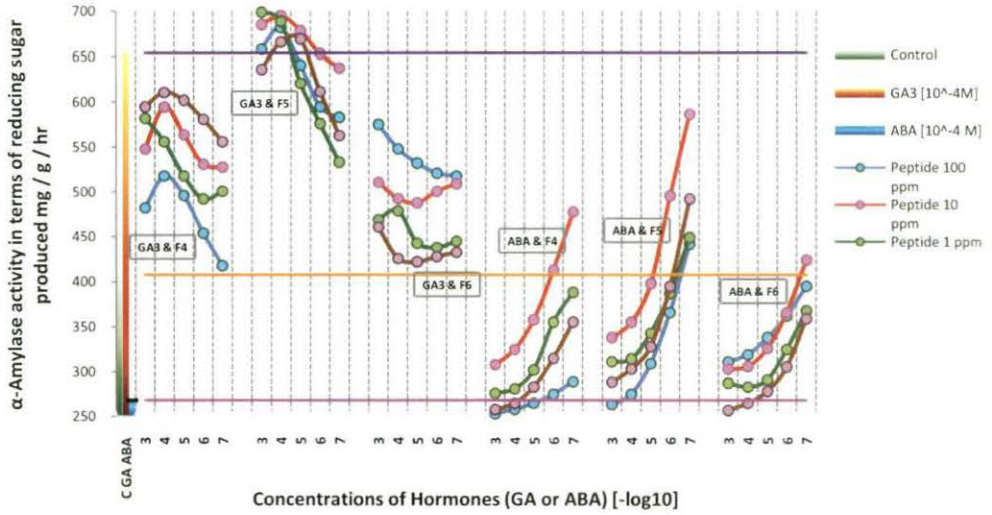


Figure 5.6 Interaction of rice LH-20 fractions F_4, F_5 and F_6 at four specified applied doses with either 10^{-4}(M) GA_3 or ABA on amylase induction or repression in incubated embryoless half barley seeds

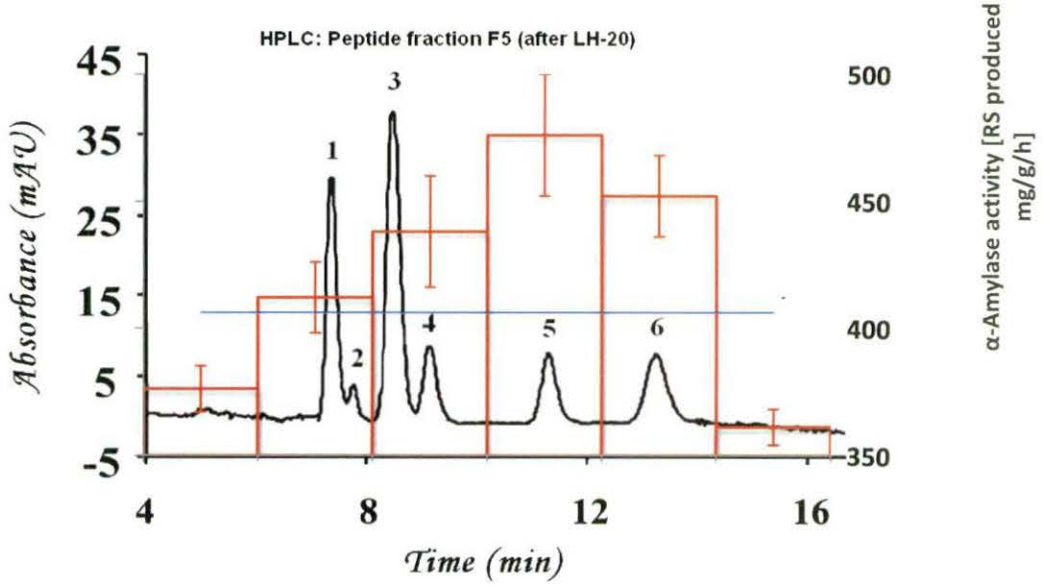


Figure 5.7 HPLC chromatogram of peptide fraction F5 and the capability of amylase induction of different peaks separated after elution

fraction was only bioactive at high concentration and the sensitivity of this fraction was disappeared at or below 1 ppm applied doses (Figure 5.5). Fraction F_4, on the other hand, represents itself in between F_5 and F_6 in terms of amylase inducing properties and this response was restored up to 10 ng/ml of peptide treatment in barley seeds (Figure 5.5).

For determining the synergistic or antagonistic interaction of LH-20 purified bioactive peptide fractions (F_4, F_5 and F_6) with gibberellin or abscisic acid, different concentration of peptides were separately applied with different doses of phytohormones. Peptide fractions were applied with four specified concentrations (100, 10, 1 and 0.1 ppm) and their interactions with five different hormone concentrations [10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} (M)] were separately studied. In case of GA_3 and F_4 interaction, better amylase induction was obtained from 10^{-4} (M) GA_3 with relatively lower doses of peptides (Figure 5.6). When the peptides from F_5 fraction were interacted with GA_3, with all peptide concentrations and interacted GA_3 in between 10^{-4} to 10^{-5} (M) treatment, enhancement of amylase induction was recorded which is more than the activity of 10^{-4} (M) GA_3 alone (Figure 5.6). F_6 peptides, on the other hand, reduced the amylase inducing activity of GA_3 during interaction; still the induction was significantly higher than control (Figure 5.6). During ABA and peptide interaction, peptides in most cases could able to suppress the ABA induced inhibition of amylase induction. Suppression of inhibition was more prominent with dilution of ABA doses and enhancement of applied peptide concentrations (Figure 5.6). Most antagonistic interaction with ABA was found with the application of 10 ppm peptide fractions of F_4, F_5 and F_6, where induction instead of inhibition, exceeds more than that of control.

With bioassay guided purification through LH-20 columns, it was observed that highest level of amylase induction in barley aleurone layers was achieved by incubating peptides from F_5 fraction. Further purification with LH-20 F_5 fraction was performed through C_{18} based reverse phase column of HPLC. HPLC chromatogram of F_5 fraction represents 6 peaks with significant absorbance at 241 nm in between 6 to 14 min retention time (Figure 5.7). This indicates that at least 6 different peptides are present in F_5 fraction. When amylase induction assay was performed by separating the peaks after elution from HPLC column, best observed induction of amylase was associated with peak number 5 (Figure 5.7). The two sharp peaks (peak number 1 and 3) with high absorbance

value as appeared in chromatogram didn't produce expected results in terms of amylase induction. In all cases, the specific activity (*i.e.* amount of induction per milligram of extracts) of peptides representing peak number 1 to 6 was decreased after HPLC based purification. It may happen that the peptides of one or more peaks synergistically act to perform amylase induction even better than GA_3 during incubation with F_5 fractions after LH-20 which may not act so efficiently after HPLC-based separation. The role of heterotrimemeric G-protein in the induction of α-amylase gene expression was established in wild oat aleurone protoplast and this G-protein may interact with different compounds and mastoparan-like peptides during regulation of GA_3 mediated signals (Jones *et al.*, 1998). Germination induced oligopeptides might interact with the receptors or intermediate proteins associated with this signal and different peptides may perhaps co-ordinate the signals of amylase induction synergistically. Unfortunately very little amount of peptides (< 1 mg) associated with peak 5 was obtained from HPLC elution profile; hence it was not possible for us to proceed through further bioassay-guided purification for ultimate identification of bioactive peptide sequence.

5.3.2 Stomatal guard cell regulation

Regulation of stomatal guard cell aperture is an osmotic phenomenon that depends upon solute accumulation in the guard cells. Light mediated stomatal opening in epidermal strips of *Commelina communis* is dependent on activation of inward potassium ion pump and subsequent accumulation of this ion in guard cells (Sawhney and Zelitch, 1969). Reports are also available where voltage dependent potassium channel and hyper-polarization of proton pumps has been recognized as basic mechanisms for controlling stomatal movement (Schroeder, 1988). Earlier findings suggested that elevation of cytosolic calcium in guard cells inhibits inward rectifying K^+ channels, which leads to voltage dependent depolarization of conductance and triggers stomatal closure (Schroeder and Hagiwara, 1989). In rice cells, the alteration of membrane potential and fluxes of proton and potassium through the plasma membrane are all transient phenomena as established in suspension culture with ^{31}P nuclear magnetic resonance spectroscopy. Recent studies indicated that different synthetic as well as natural peptides

Table 5.2 Percentage of stomatal opening and guard cell aperture width in light and dark after treatment with oligopeptides and abscisic acid

Time of obs., treatment and conditions	*Sl. No. of observed microscopic fields*	*No. of stomata observed per microscopic field*	*Number of opened stomata*	*Percentage of opened stomata*		*Width of aperture (μm)*	
				Each field	*Mean ± SD*	*Each field*	*Mean ± SD*
Immediately after peeling	I	18	4	16.67	**38.25 ± 12.90**	3.35	**3.47 ± 0.76**
	II	12	4	25.00		3.45	
	III	14	6	42.86		4.29	
	IV	14	8	57.14		2.29	
	V	14	5	714		3.96	
	Total:	**72**	**27**	**37.50**		-	
Exposure to bright sunlight for 2 hrs.	I	21	11	52.38	**56.04 ± 11.71**	3.82	**3.99 ± 0.47**
	II	14	9	64.29		3.32	
	III	14	10	71.43		3.96	
	IV	19	8	42.11		4.29	
	V	20	10	50.00		4.56	
	Total:	**88**	**48**	**54.55**		3.82	
Placed in dark for 2 hrs.	I	20	1	5.00	**3.83 ± 2.17**	0.82	**0.56 ± 0.39**
	II	17	0	0.00		0	
	III	23	1	4.35		0.56	
	IV	19	1	5.26		0.42	
	V	22	1	4.55		1.02	
	Total:	**101**	**4**	**3.96**		-	
2 hrs. light exposure + heterogeneous oligopeptides [10 μg / ml]	I	20	8	50.00	**41.23 ± 5.24**	3.67	**3.94 ± 0.45**
	II	15	7	46.67		4.12	
	III	17	6	35.29		4.56	
	IV	15	7	60.00		3.38	
	V	16	6	50.00		3.97	
	Total:	**83**	**34**	**40.96**		-	

Table 5.2 Contd.

Time of obs., treatment and conditions	*Sl. No. of observed microscopic fields*	*No. of stomata observed per microscopic field*	*Number of opened stomata*	*Percentage of opened stomata*		*Width of aperture (μm)*	
				Each field	*Mean ± SD*	*Each field*	*Mean ± SD*
2 hrs. dark + heterogeneous oligopeptides [10 μg / ml]	I	21	8	38.10	**38.51 ± 2.44**	3.67	**3.48 ± 0.32**
	II	15	6	40.00		3.12	
	III	12	5	41.67		3.56	
	IV	17	6	35.29		3.18	
	V	16	6	37.50		3.87	
	Total:	**81**	**31**	**38.27**		-	
2 hrs. light exposure + ABA [10^{-5} M]	I	18	1	5.56	**4.57 ± 2.65**	0.67	**0.72 ± 0.44**
	II	21	1	4.76		1.12	
	III	13	0	0.00		0	
	IV	17	1	5.88		0.88	
	V	15	1	6.67		0.95	
	Total:	**84**	**4**	**4.76**		-	
2 hrs. dark + ABA [10^{-5} M]	I	15	0	0.00	**0.00 ± 0.00**	0.00	**0.00 ± 0.00**
	II	14	0	0.00		0.00	
	III	18	0	0.00		0.00	
	IV	20	0	0.00		0.00	
	V	16	0	0.00		0.00	
	Total:	**83**	**0**	**0**		-	

could alter cytoplsmic pH and calcium level in guard cells of different plant species and modulate stomatal opening. Previous work has shown that the C-terminal dodecapeptide of auxin binding protein (ABP1) induced guard cell alkalinisation and stomatal closure in epidermal strips of orchid *Paphipedilum tonsum* L. (Gehring *et al.*, 1998). This oligopeptide is highly charged and unable to permeate the plasma membrane and thus bind to the docking site of receptor protein and triggers proton extrusion and stomatal closure (Thiel *et al.*, 1993). Naturally occurring wound signal induced oligopeptide systemin was also found to block proton pump as established in cell suspension of *Lycopersicon peruvianum* and this proton pump inhibition was abolished when calcium influx was blocked (Schaller and Oecking, 1999). On the other hand, calcium influx in parsley suspension cells may be mediated through an oligopeptide elicitor derived from cell wall of phytopathogenic fungus *Phytophthora sojae* (Zimmermann *et al.*, 2004). Studies on guard cells of maize have shown that external application of auxin modulated the voltage gating of anion channels even in excised membrane patches and extracellularly applied antibody raised against synthetic peptide with sequence similarity of active site of auxin binding protein mimic auxin action and shifted the action potential like naphthalene acetic acid (NAA) (Venis *et al.*, 1992). Also the peptides are reported to play an important role in downstream signal of calcium ion. Ca^{2+}-dependent SOS2 protein of *Arabidopsis thaliana* can phosphorylate both serine-containing and threonine-containing synthetic peptides having recognition sequence of protein kinase-C and by this way the K^+ ion homeostasis in plant cells were maintained (Halfler *et al.*, 2000). In guard cell membranes of *Vicia faba*, the existence of G-protein linked 7TMS receptors were established and amphipathic tetradecapeptide mastoparan (Mas7) activated that G-protein receptor, and modulate the inward rectifying K^+ ion channels of guard cells (Blatt and Armstrong, 1993). All the above mentioned phenomena indicated that peptides, either natural or synthetic origin may modulate ionic homeostasis and participate in stomatal guard cell aperture regulation either directly or indirectly.

In this investigation, the effect of isolated rice peptides on stomatal opening and guard cell aperture width was measured in both light and dark conditions. As shown in Table 5.2, after immediate peeling, 38.25% stomata were opened, but the opening might enhance up to 56.04% after 2 hrs exposure under bright sunlight without any change in

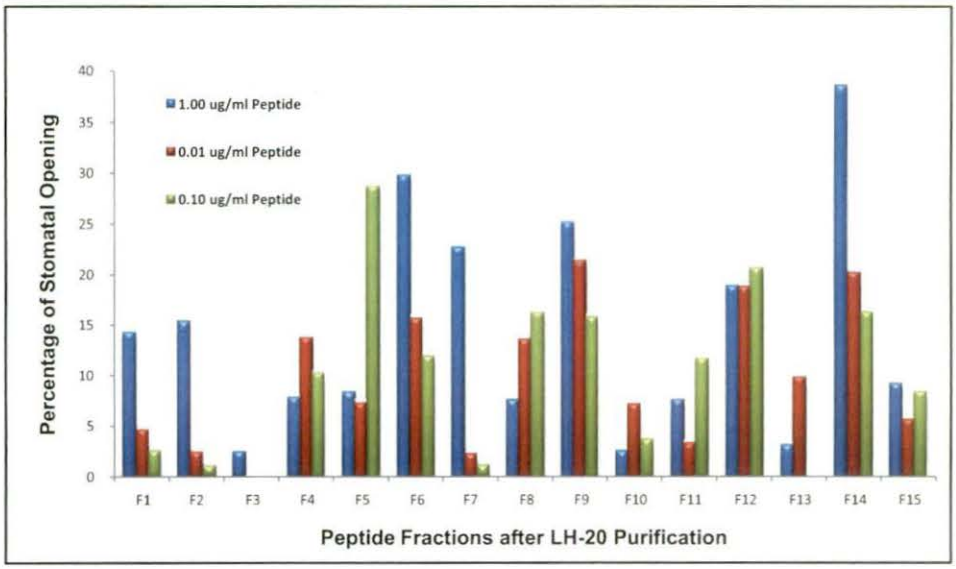


Figure 5.8 Bar diagram showing effect of different peptide concentration after LH-20 fractionation on stomatal opening in dark

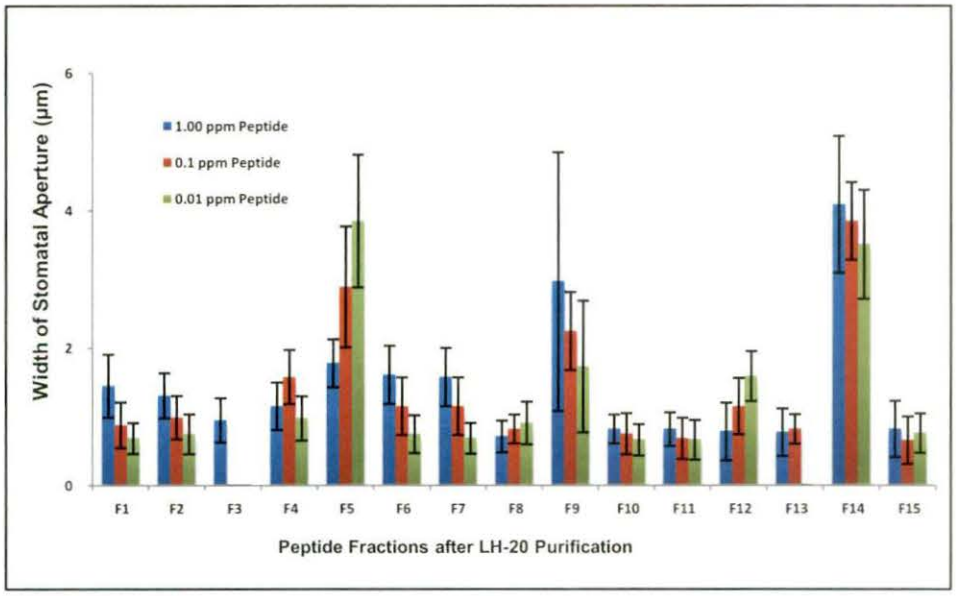


Figure 5.9 Bar diagram showing effect of different peptide concentration after LH-20 fractionation on width of guard cell aperture in dark

Table 5.3 Effect of different LH-20 purified rice peptide fractions on stomatal opening and aperture width

Peptide Fraction No.	Percentage of stomatal opening in different concentrations of peptides				Width of stomatal aperture in different concentrations of peptides			
					Range of aperture width (µm)	Mean value with standard deviation (µm)		
	Control	1 g / ml	1 x 10^{-2} g /ml	1 x 10^{-4} g /ml		1 g / ml	1 x 10^{-2} g /ml	1 x 10^{-4} g /ml
F1	0	14.28	4.65	2.56	0.66-1.65	1.45±0.457	0.88±0.331	0.69±0.225
F2	0	15.38	2.43	1.08	0.66-1.32	1.31±0.328	0.99±0.314	0.75±0.288
F3	0	2.46	0.00	0.00	0.66-1.32	0.95±0.322	0.00	0.00
F4	0	7.82	13.71	10.22	0.66-1.65	1.155±0.345	1.58±0.391	0.98±0.322
F5	0	8.33	7.29	28.73	3.33-4.29	1.78±0.348	2.89±0.882	3.85±0.968
F6	0	29.8	15.7	11.9	0.66-1.65	1.61±0.422	1.155±0.419	0.75±0.275
F7	0	22.7	2.3	1.12	0.66-1.65	1.58±0.422	1.155±0.419	0.69±0.226
F8	0	7.52	13.53	16.2	0.66-0.99	0.716±0.231	0.825±0.211	0.911±0.312
F9	0	25.2	21.4	15.8	1.65-3.96	2.97±1.882	2.245±0.565	1.73±0.958
F10	1.53	2.52	7.21	03.70	0.66-0.99	0.825±0.211	0.755±0.298	0.667±0.228
F11	0	7.56	3.26	11.66	0.66-0.99	0.825±0.245	0.688±0.299	0.667±0.288
F12	0	18.90	18.8	20.69	0.66-1.65	0.788±0.421	1.155±0.411	1.592±0.361
F13	0	3.11	9.87	0.00	0.66-0.99	0.778±0.345	0.825±0.216	0.00
F14	0	38.56	20.22	16.20	3.35-4.29	4.09±0.988	3.85±0.968	3.51±0.994
F15	0	9.24	5.56	8.42	0.66-0.99	0.825±0.411	0.661±0.344	0.768±0.288

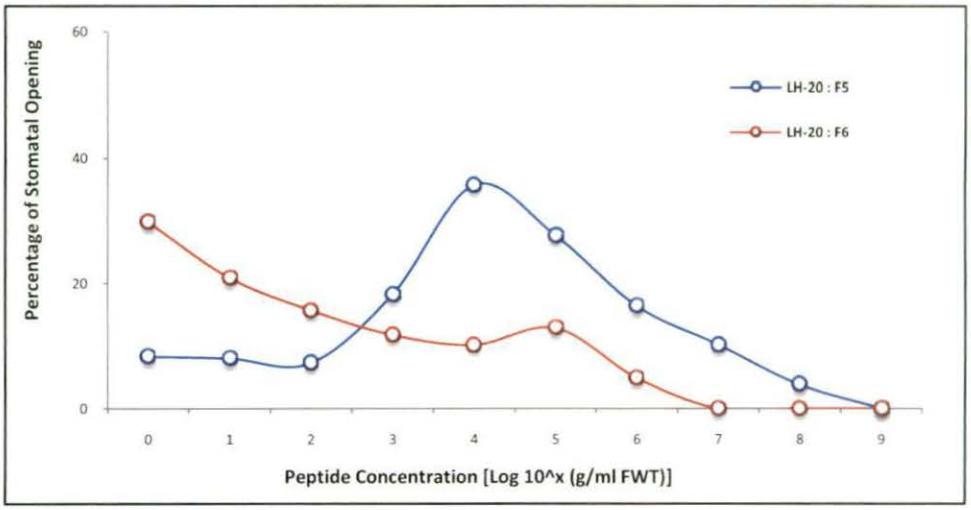


Figure 5.10 Effect of various concentrations of LH-20 purified peptide fraction F_5 and F_6 on percentage of stomatal opening in dark

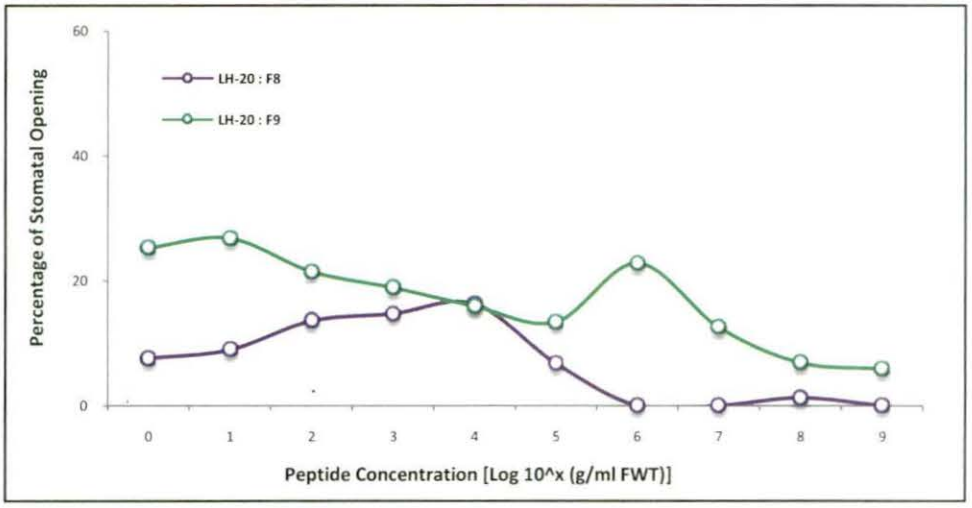


Figure 5.11 Effect of various concentrations of LH-20 purified peptide fraction F_8 and F_9 on percentage of stomatal opening in dark

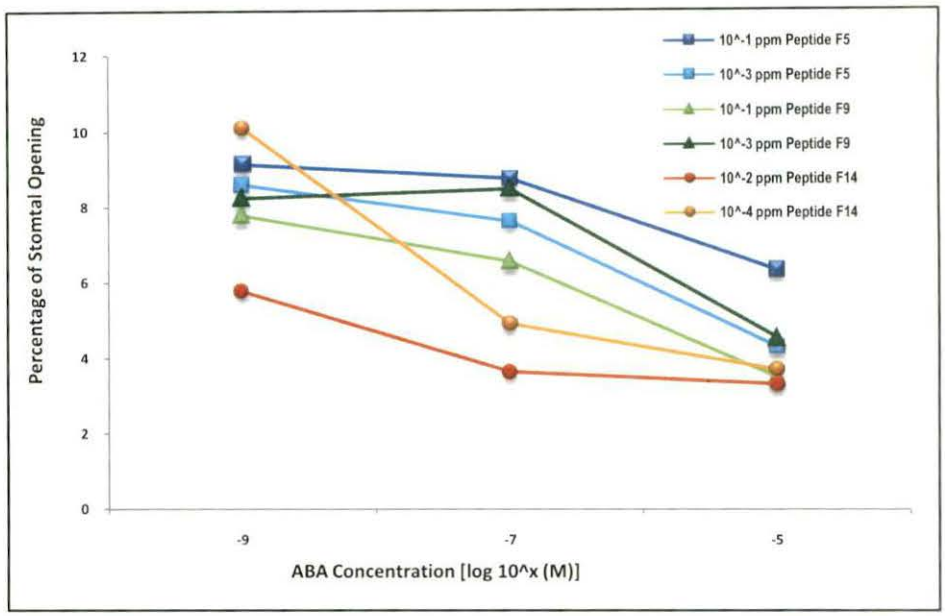


Figure 5.12 Effect of various concentrations of bioactive peptide fractions after LH-20 purification on percentage of stomatal opening and their interaction with different doses of ABA

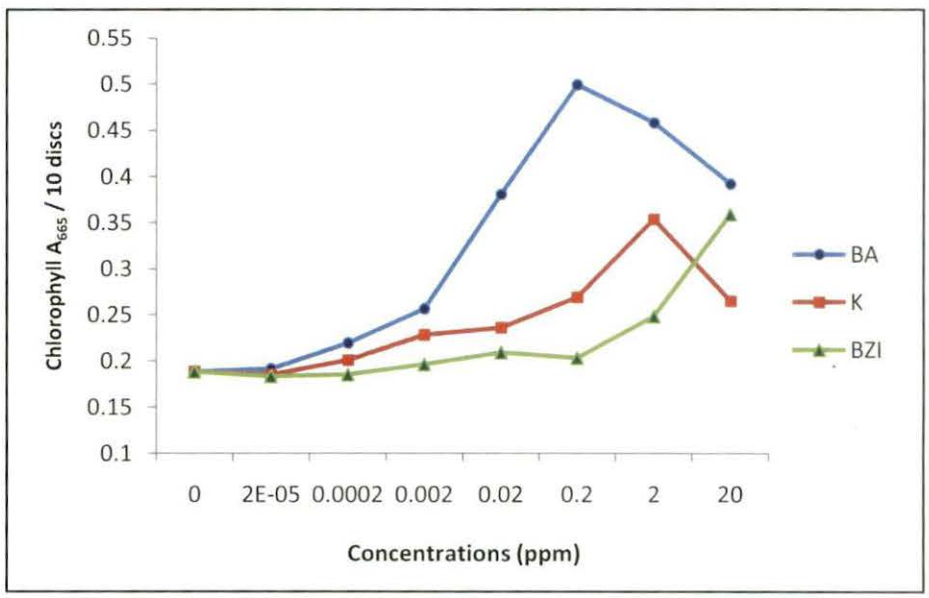


Figure 5.15 Chlorophyll retention activities of BA, K and BZI in leaf discs of radish (*Raphanus sativus*)

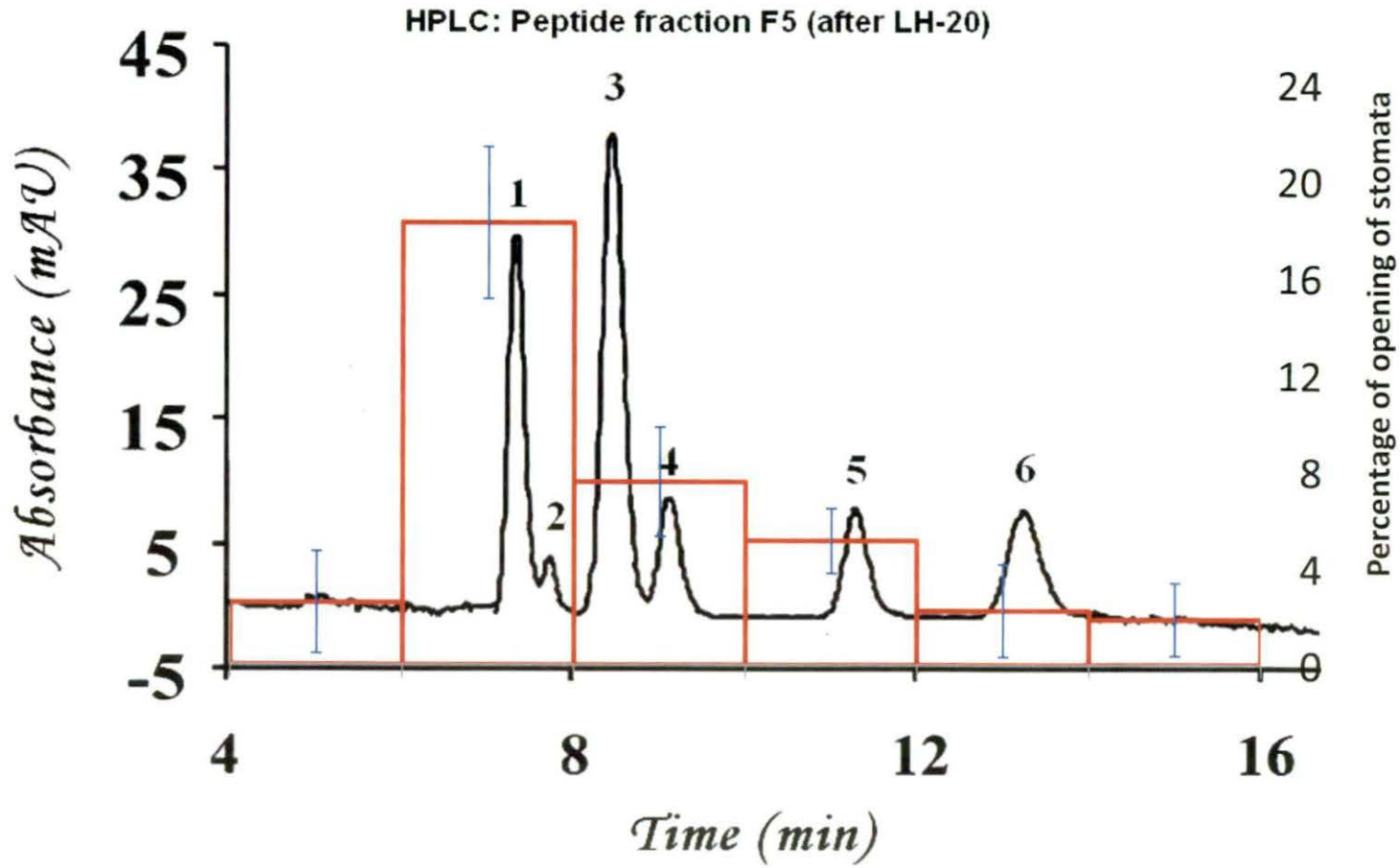


Figure 5.13 HPLC profile of rice peptide LH-20 F_5 fraction and relative response of different peaks on stomatal opening

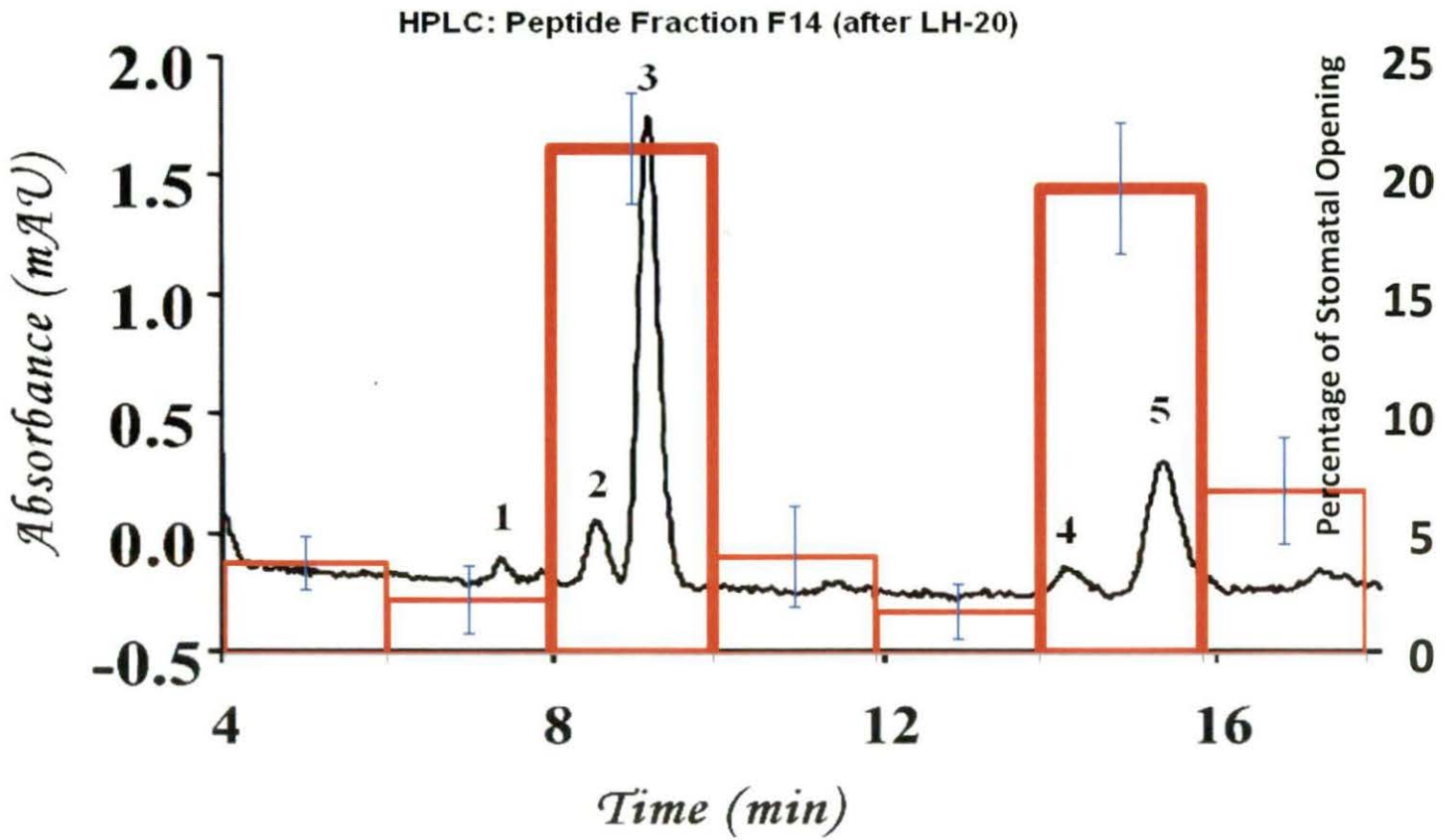
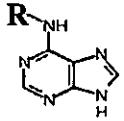
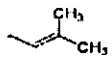
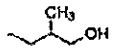
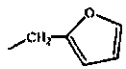
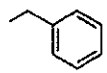


Figure 5.14 HPLC profile of rice peptide LH-20 F_{14} fraction and relative response of different peaks on stomatal opening

width of aperture. When the same epidermal layers were placed for 2 hrs in dark, percentage of opened stomata were reduced up to 3.83% along with decrease in aperture width (0.56 μm) (Table 5.2). Two hours exposure under sunlight with heterogeneous oligopeptides (10μg/ml) didn't enhance stomatal opening (41.23%) beyond control. However, when the same concentration of peptides was applied with 2 hrs dark phase, percentage of opened stomata (28.51%) was significantly higher (almost 10 times) than control (i.e. only in dark). Not only that, guard cell aperture width was also remarkably enlarged (almost 6.2 times) after peptide treatment in dark, when compared with untreated epidermal peelings in dark (Table 5.2). In contrast, ABA could able to close stomata in light and dark up to 95% and 100% respectively (Table 5.2).

When the percentage of stomatal opening was measured in dark with the peptides of LH-20 purified fractions at three specified concentrations (1, 0.1 and 0.01 ppm) on epidermal strips of *Commelina benghalensis*, significant opening of stomata were found in F_5, F_6, F_8, F_9, F_{12} and F_{14} fractions, but highest activity was achieved by 1 ppm of F_{14} peptide treatment (Figure 5.8). With same concentration width of stomatal aperture was increased with the application of F_5, F_9 and F_{14} peptide fractions (Figure 5.9). As shown in Table 5.3, application of peptides with higher concentration didn't always produce fruitful results in different fractions. Among different LH-20 purified fractions, three bioactive fractions (F_5, F_9 and F_{14}) were chosen for determining their interaction with antagonistic hormone ABA, which is responsible for stomatal closure. In all cases, abscisic acid could able to suppress the stomatal opening significantly, particularly at higher concentrations (Figure 5.12). When the concentration of peptide doses were increased, inhibition of stomatal opening by ABA was to some extent reverted (Figure 5.12). Maximum inhibition by ABA was observed on 10^{-9} ppm peptide doses of F_{14}, whereas this inhibition was minimized with 10^{-1} ppm peptide application of F_5 fraction (Figure 5.12). Percentage of stomatal opening was also measured on epidermal strips with various concentrations (10^{-1} to 10^{-9} ppm) of peptide treatment available in bioactive fraction F_5, F_6, F_8 and F_9 for calculating their optimal doses (Figure 5.10 and 5.11). At 10^{-4} ppm fraction, F_5 exhibited maximum opening of stomata (Figure 5.10), whereas the fraction F_9 produced almost same response at 10^{-6} ppm concentration (Figure 5.11). Other two fractions (F_6 and F_8) didn't respond significantly at lower concentrations.

Table 5.4 Chemical structure and abbreviations of different cytokinins used for chlorophyll retention in radish leaf discs

Name	***Abbreviations***	***Structure***
Cytokinins		
		R=
Isopentenyl adenine	IPA	
Zeatin	Z	
Dihydrozeatin	DHZ	
Kinetin	K	
Benzyl adenine	BA	
Benzymidazole	BZI	

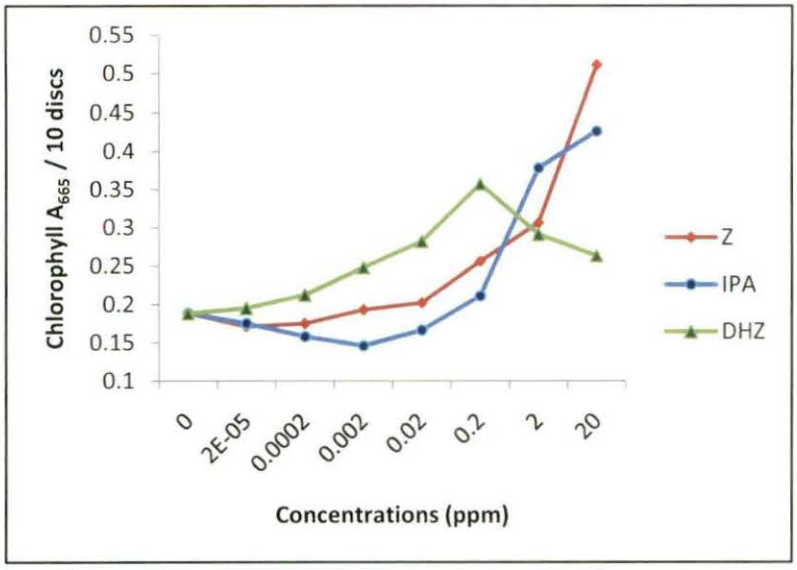


Figure 5.16 Chlorophyll retention activities of Z, IPA and DHZ in leaf discs of radish (*Raphanus sativus*)

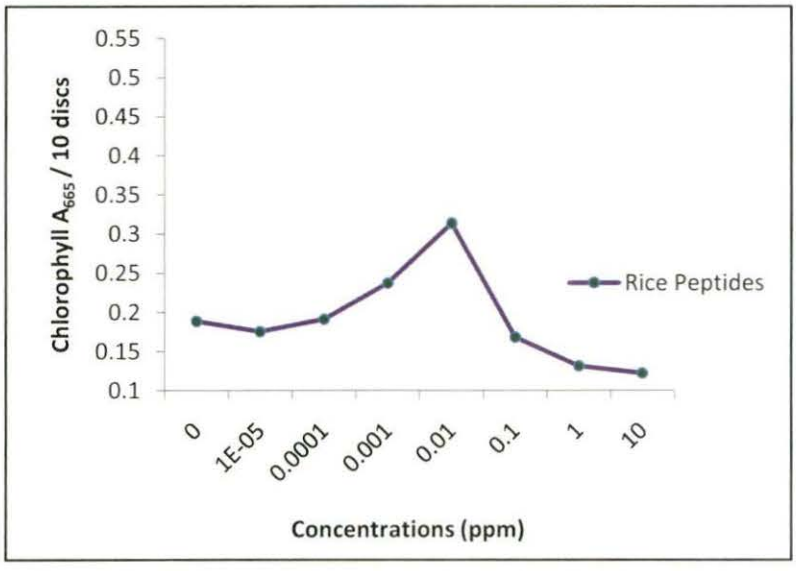


Figure 5.17 Chlorophyll retention activities of isolated rice peptides in leaf discs of radish (*Raphanus sativus*) mimic cytokinins action

For determining exact number of peptides in different LH-20 purified fractions which were involved in stomatal guard cell regulation, HPLC analysis of bioactive fraction F_5 and F_{14} were performed by using C_{18} (octadecasilane) reverse phase column and methanol as polar mobile phase for elution. Six different peaks were obtained from F_5 fraction with high absorbance value at 214 nm (Figure 5.13). Percentage of stomatal opening in dark was also measured with eluted volume after removal of mobile phase collected from HPLC column at fixed time interval. Best dark incubated stomatal opening response was associated with peak 1 and 2, whereas in case of other peaks, percentage of opening of stomata was very low (Figure 5.13). From peptide fraction F_{14}, five different peaks were obtained of which peak number 3 gathered maximum peak area with high absorbance value (Figure 5.14). Stomatal opening response was also determined with eluted volume and significant percentage of stomatal opening was associated with peak 3 and 5 (Figure 5.14). Further analysis and sequence determination of these peaks were not performed due to shortage of time.

5.3.3 Retention of chlorophyll

During senescence, the catabolic pathway of chlorophyll is mainly regulated by three prime enzymes: Chlorophyllase, Pheophorbide-*a* oxygenase and chlorophyll catabolite reductase (Hortensteiner, 2006). Chlorophyll breakdown is a prerequisite for detoxification of phototoxic pigments through which chlorophyll binding proteins are processed for remobilization of nitrogen in fresh tissue (Hortensteiner and Feller, 2002). Previous reports claimed that plant hormone cytokinins retard chlorophyll degradation very efficiently (Yu and Kao, 1981). As shown in Table 5.4, phytohormone cytokinins may categorized into three main classes: (i) cytokinins with straight chains attached at sixth nitrogen position of adenine like Isopentenyl adenine (IPA), Zeatin (Z) and Dihydrozeatin (DHZ); (ii) cytokinins with ring structures at sixth nitrogen position of adenine such as Benzyl adenine (BA) and Kinetin (K); and (iii) cytokinin without adenine moiety like Benzimidazole (BZI). Chlorophyll retention activities of different cytokinins in radish leaf discs were represented in Figure 5.15 and 5.16. The chlorophyll retention of various cytokinins with straight side chain decreased in radish leaf discs in the following order: DHZ > Z > IPA below 0.2 ppm concentrations (Figure 5.16). At

Table 5.5 Retardation of chlorophyll disappearance in radish leaf discs after application of LH-20 purified peptide fractions and Benzyl adenine (BA) as standard

Peptide Fraction No.	Retardation of chlorophyll disappearance (after 96 h ageing)							
	Radish leaf discs (E_{665}/10 leaf discs) [Peptide concentrations]				Mean % of chlorophyll retention [Peptide concentrations]			
	100 ppm	10 ppm	1 ppm	0.1 ppm	100 ppm	10 ppm	1 ppm	0.1 ppm
Initial State			779				-	
Control			182				23.36	
BA (10^{-5} M)			**526**				**67.52**	
F_1	194 ± 16	216 ± 21	**239 ± 18**	**256 ± 15**	24.90	27.73	**30.68**	**32.86**
F_2	159 ± 23	162 ± 17	178 ± 21	165 ± 15	20.41	20.80	22.85	21.18
F_3	172 ± 22	215 ± 13	**239 ± 16**	**263 ± 18**	22.08	27.60	**30.68**	**33.76**
F_4	191 ± 25	**244 ± 22**	207 ± 18	198 ± 18	24.52	**31.32**	26.57	25.42
F_5	**221 ± 23**	**256 ± 22**	218 ± 24	195 ± 26	**28.37**	**32.86**	27.98	25.03
F_6	186 ± 14	202 ± 16	**254 ± 18**	**291 ± 20**	23.88	25.93	**32.61**	**37.36**
F_7	188 ± 19	**228 ± 15**	**241 ± 14**	**303 ± 22**	24.13	**29.27**	**30.94**	**38.90**
F_8	185 ± 12	178 ± 14	164 ± 18	162 ± 12	23.75	22.85	21.05	20.80
F_9	191 ± 14	183 ± 14	176 ± 18	161 ± 24	24.52	23.49	22.59	20.67
F_{10}	195 ± 15	202 ± 18	218 ± 14	205 ± 22	25.03	25.93	27.98	26.32
F_{11}	165 ± 22	176 ± 23	168 ± 21	178 ± 14	21.18	22.59	21.57	22.85
F_{12}	188 ± 16	191 ± 16	196 ± 14	192 ± 12	24.13	24.52	25.16	24.65
F_{13}	218 ± 15	**234 ± 18**	**272 ± 18**	**307 ± 14**	27.98	**30.04**	**34.92**	**39.41**
F_{14}	207 ± 22	**235 ± 24**	**221 ± 20**	181 ± 18	26.57	**30.17**	**28.37**	23.23
F_{15}	163 ± 16	158 ± 14	169 ± 16	152 ± 18	20.92	20.28	21.69	19.51

higher concentrations of DHZ, retardation of chlorophyll activity were minimized (Figure 5.16). Zeatin (Z) on the other hand, retards chlorophyll more efficiently at higher applied doses. With cytokinins ring structures at the sixth nitrogen position of adenine, the effect of retarding chlorophyll degradation was BA > K below 0.2 ppm applied doses (Figure 5.15). In both BA and K, 0.2 ppm was found to be optimal in nature. At 0.2 ppm, BA exhibited 269.4% higher retention capacity than control, which is also superior to the activity of other cytokinins at same concentration. BZI at or below 0.2 ppm concentrations didn't perform chlorophyll retention activity, indicating that adenine moiety is required for cytokinins action (Figure 5.15). From earlier records, it may be stated that urea derivatives with substituted nitro-phenyl ring structure efficiently retard chlorophyll in radish leaf disc and leaf senescence at 10^{-6} (M) concentration (Kefford *et al.*, 1973). Plant euginols with 6-substituted phenol can also retard chlorophyll disappearance in radish leaf discs (Karanov *et al.*, 1995). Different transit peptides were discovered recently from chloroplast and thylakoids of eukaryotes, but their role in chlorophyll synthesis and degradation are still obscure (Turkina *et al.*, 2004). Chlorophyll retention capacity of isolated rice peptides optimized at 0.01 ppm applied doses which is remarkably 166.49% higher than control (Figure 5.17). But when compared with other cytokinins' action, this activity was not looking very significant at their optimized doses. At higher peptide doses (≥ 1 ppm), the rate of degradation of chlorophyll was accelerated more than control (Figure 5.17). After LH-20 purification, evaluation of disappearance of chlorophyll in radish leaf discs was continued for determining senescence retardation and cytokinins like action of specific peptides from isolated rice homogenates after ultrafiltration. Here BA (10^{-5} M) was used as standard and 67.52% chlorophyll retention was observed from initial state after BA application (Table 5.5). It was observed that chlorophyll retention activity of peptides were distributed in F_1, F_3, F_4, F_5, F_6, F_7, F_{13} and F_{14}, when applied on radish leaf discs at four specified concentrations (100, 10, 1 and 0.1 ppm). Fractions F_1, F_3 and F_6 were partially bioactive only at lower concentrations (≤ 1 ppm) whereas the same activity was more prominent at higher concentrations in F_5 and F_{14}, however in all cases nearly about 30% of chlorophyll was restored from initial which indicates that the results were not very significant (<10% high) in respect to control (Table 5.5). Further bioassay guided purification of these peptide fractions were not

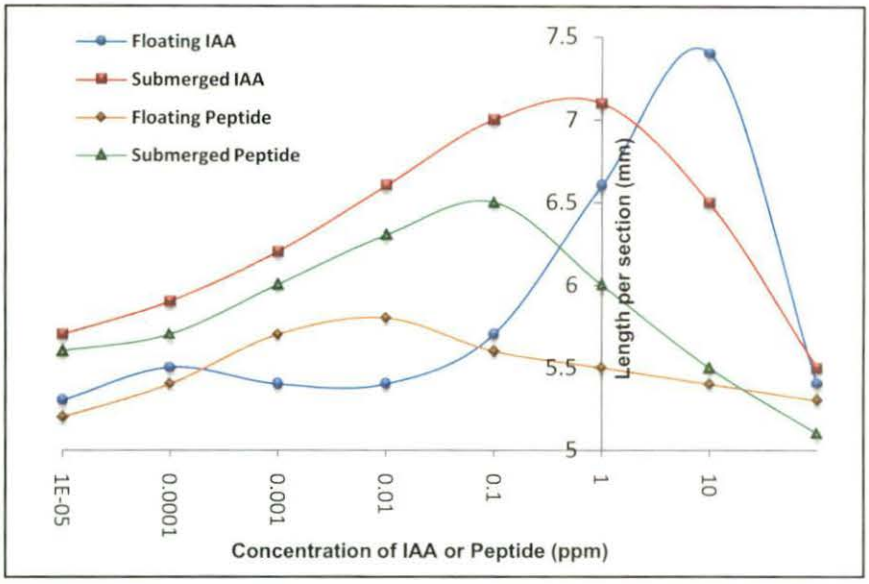


Figure 5.18 Coleoptile elongations of submerged and floating sections with IAA and peptide treatment

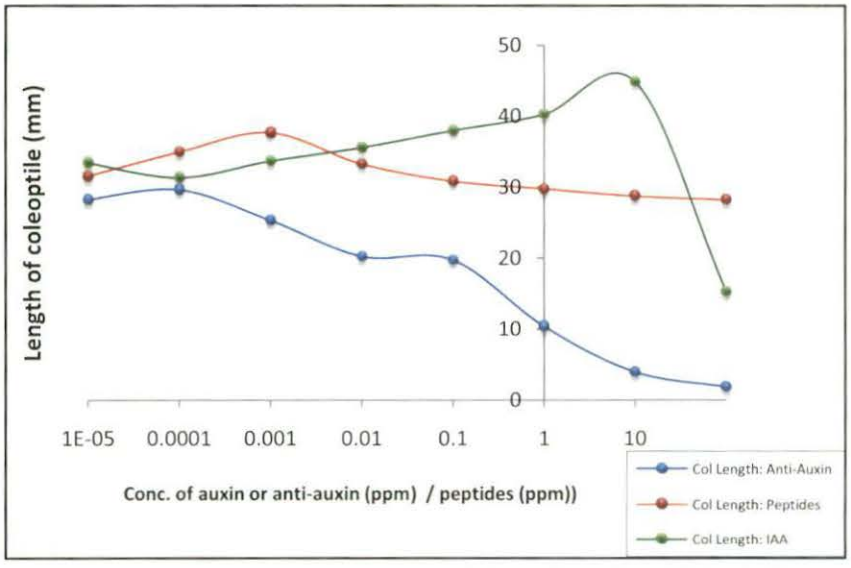


Figure 5.19 Coleoptile lengths in intact seedlings after application of anti-auxin, peptides and IAA

performed because the initial activity was distributed and no fraction was found to be remarkably higher than control or as per the results of cytokinins standard used in this experiment.

5.3.4 Elongation of coleoptile

In rice, maximum coleoptile elongation rate was achieved under oxygen tension (Nagao and Ohwaki, 1953). This phenomenon was related with IAA oxidases activity when the coleoptile was not submerged in water (Yamada, 1954). The optimal concentration of IAA is higher in submerged sections, when compared floated counterparts. Reports are also available where guaiacol promoted the growth of rice coleoptile even in presence of oxygen, as the substance inhibited IAA oxidase system *in vitro* (Wada and Nagao, 1960). From the evolutionary viewpoint, under anoxia successful coleoptile growth provides rice seedlings with an opportunity to reach above anaerobic mud or standing water, thus increasing the chance of survival (Kordan, 1974). This dramatic response of rice coleoptiles under anoxia is associated with accumulation of specific amino acids and enhanced level of altered metabolite pools (Shingaki-Wells *et al.*, 2011). It was revealed that the morphological changes of rice seedlings was highly correlated with peptide-N-glycanase (PNGase) activity of imbibed rice grains, though the function of this enzyme-catalyzed deglycosylation during post-germinative development is unknown (Chang *et al.*, 2000). It may happen that PNGase catalyzed oligopeptides produce chemical signals, which mimic auxin action in developing rice coleoptiles. Coleoptile growth of rye seedlings (rice allied family) was also associated with upregulation of specific peptide sequences, particularly related with subunit-E of vacuolar H^+-ATPase (Kutschera *et al.*, 2010). In this investigation, when IAA was applied on floating and submerged coleoptile sections, maximum coleoptile growth was observed at lower dilution of IAA during submergence, whereas for attaining the same coleoptile growth in case of floating sections, higher applied concentrations of IAA were necessary (Figure 5.18). This indicates that exogenous IAA was either more sensitive to submerged coleoptile sections or level of endogenous IAA was high in submerged sections; therefore the requirement of applied exogenous IAA was low. When the peptides were applied on submerged coleoptile sections, optimum response was observed at 0.1 ppm peptide concentration,

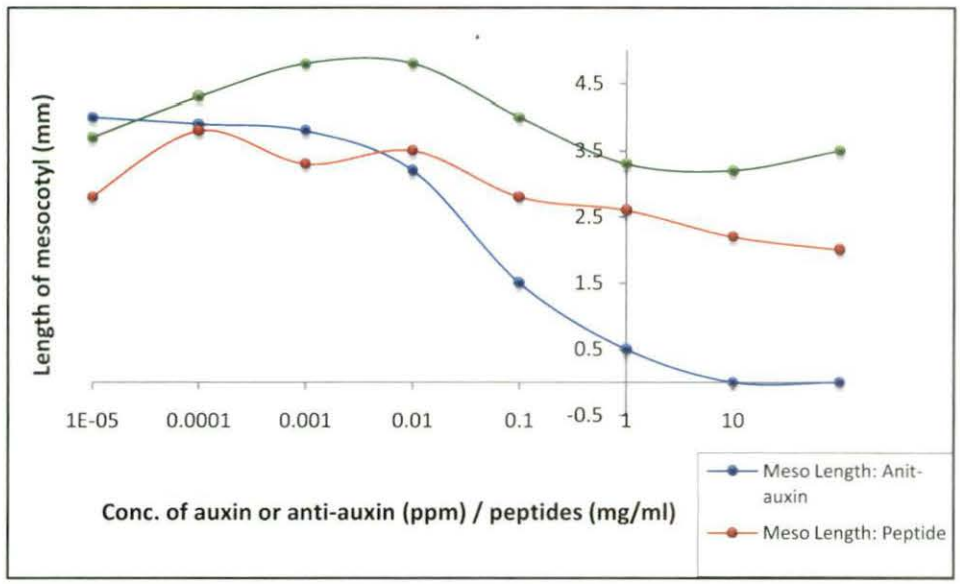


Figure 5.20 Mesocotyl lengths in intact seedlings after application of anti-auxin, peptides and IAA

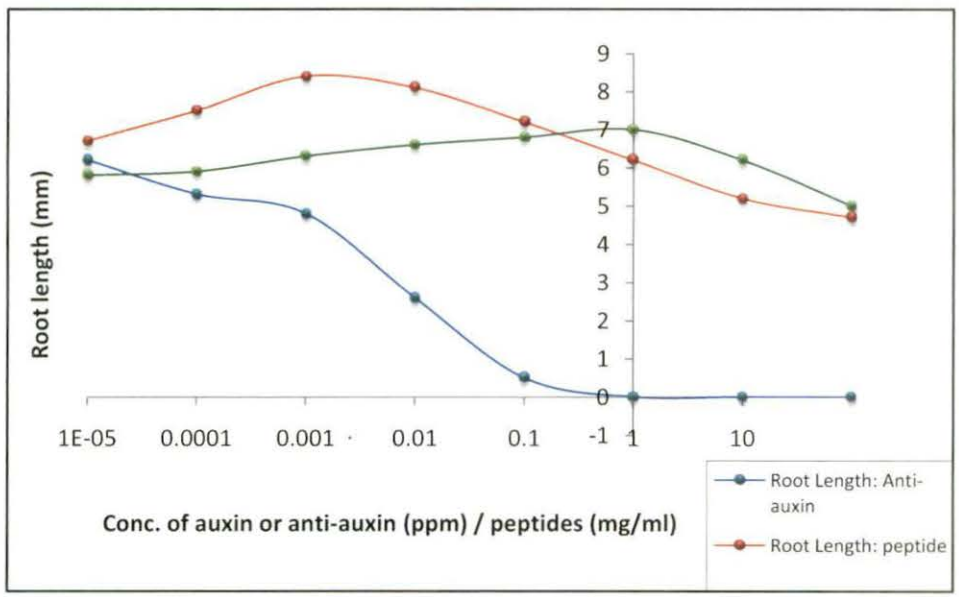


Figure 5.21 Root lengths in intact seedlings after application of anti-auxin, peptides and IAA

Table 5.6 Elongation of *Triticum aestivum* L. coleoptile segments after application of LH-20 purified rice peptide fractions and IAA as standard

Peptide Fraction No.	Elongation of *Triticum aestivum* coleoptiles segments with rice peptides							
	Elongation (mm) [Peptide concentrations]				Mean % to the control [Peptide concentrations]			
	100 ppm	10 ppm	1 ppm	0.1 ppm	100 ppm	10 ppm	1 ppm	0.1 ppm
Control	2.0 ± 0.2				100			
IAA (10^{-5} M)	5.1 ± 0.4				260			
F_1	1.8 ± 0.2	1.7 ± 0.2	1.9 ± 0.1	1.9 ± 0.2	90	85	95	95
F_2	1.7 ± 0.3	1.6 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	85	80	90	90
F_3	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.2	2.2 ± 0.3	95	95	105	110
F_4	2.2 ± 0.3	2.1 ± 0.2	2.2 ± 0.3	2.3 ± 0.3	110	105	110	115
F_5	2.3 ± 0.3	2.4 ± 0.3	2.4 ± 0.3	2.6 ± 0.2	115	120	120	130
F_6	2.3 ± 0.3	2.5 ± 0.3	2.6 ± 0.3	2.8 ± 0.4	115	125	130	140
F_7	2.2 ± 0.2	2.1 ± 0.3	2.1 ± 0.2	1.9 ± 0.2	110	105	105	95
F_8	2.0 ± 0.2	2.1 ± 0.3	2.1 ± 0.3	2.0 ± 0.3	100	105	105	100
F_9	1.9 ± 0.1	1.7 ± 0.2	1.6 ± 0.2	1.7 ± 0.3	95	85	80	85
F_{10}	2.0 ± 0.2	2.1 ± 0.2	2.1 ± 0.3	2.2 ± 0.2	100	105	105	110
F_{11}	1.9 ± 0.2	1.9 ± 0.2	1.8 ± 0.1	1.8 ± 0.2	95	95	90	90
F_{12}	1.8 ± 0.2	1.6 ± 0.3	1.5 ± 0.2	1.8 ± 0.2	90	80	75	90
F_{13}	2.1 ± 0.3	2.4 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	105	120	125	125
F_{14}	1.7 ± 0.1	2.0 ± 0.1	2.0 ± 0.2	2.3 ± 0.3	85	100	100	115
F_{15}	1.6 ± 0.2	1.5 ± 0.2	1.8 ± 0.3	1.9 ± 0.2	80	75	90	95

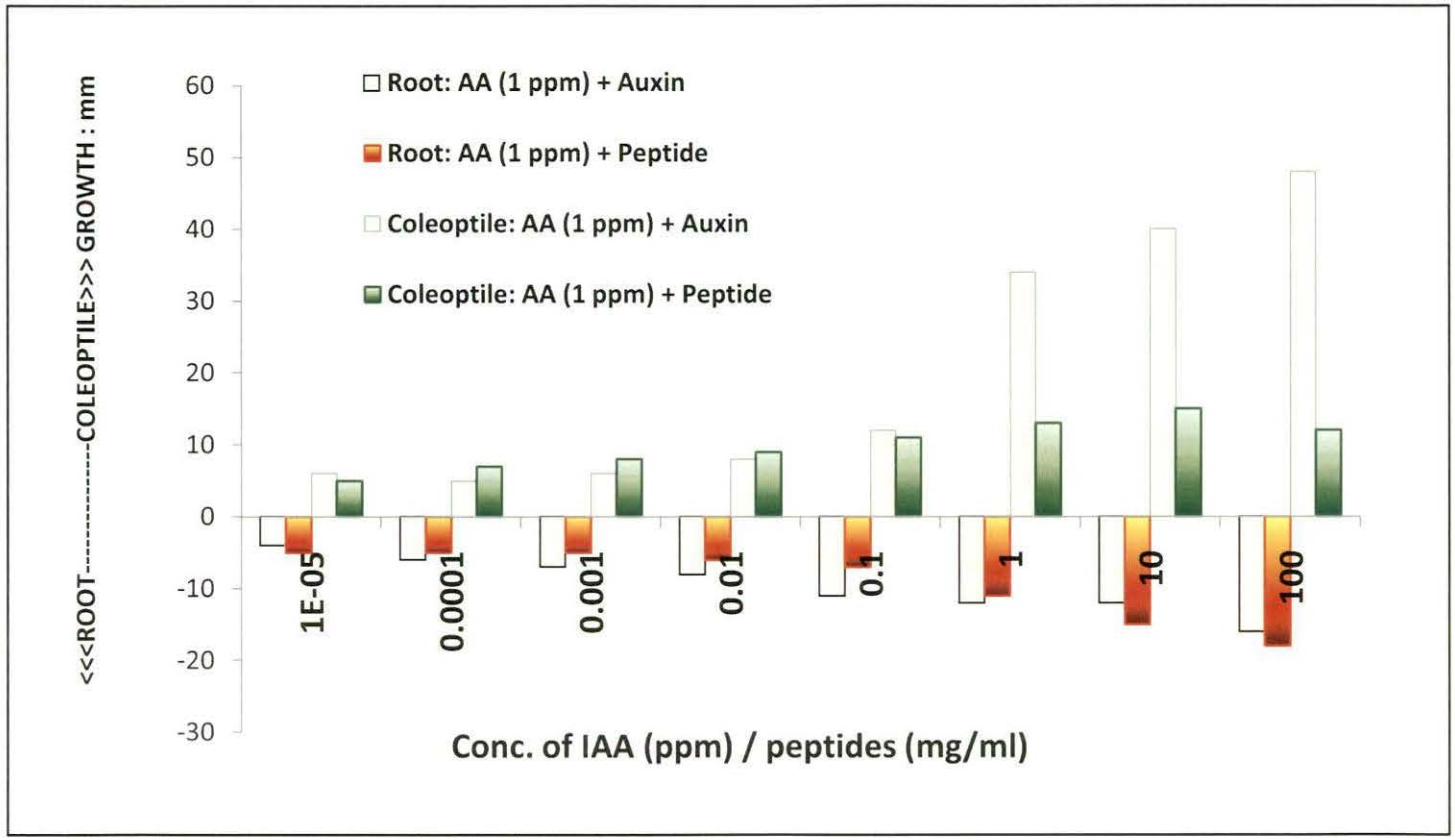


Figure 5.22 Antagonistic interactions between anti-auxin and auxin/peptide on root and coleoptile elongation of intact rice seedlings

through which coleoptile growth was attained up to 6.5 mm from 4 mm initial stage after 72 hrs incubation (Figure 5.18). In contrast, the same response of peptides on floating sections was almost insignificant. So it may be concluded that peptide can only produce bioactivity (coleoptile elongation) under hypoxic or anoxic stress, when IAA oxidase was presumably inactive. Length of coleoptile, mesocotyl and root was also measured in intact seedlings 48 hrs after application of peptides, IAA and anti-auxin (Figure 5.19-5.21). Steady decrease of length of coleoptiles, mesocotyl and roots of rice seedlings were observed with applied higher doses of anti-auxin. In case of auxin, increment of coleoptile length was optimized at comparatively higher doses; whereas the mesocotyl and roots were more sensitive to auxin, when their elongation was considered (Figure 5.19-5.21). With peptide application, drastic improvement of coleoptile and mesocotyl length was not observed, but root length was significantly developed at lower doses of peptide treatment in intact seedlings (Figure 5.21). When the antagonistic interaction between anti-auxin and auxin or peptides as auxin-agonist was measured, inhibition of coleoptile elongation was overcome by auxin at higher doses, but the peptide mediated coleoptile elongation was successfully suppressed by anti-auxin even during higher applied doses of peptides (Figure 5.22). Inhibition of root elongation by anti-auxin, on the other hand, was to some extend recovered by auxin or peptide treatment at their higher doses (Figure 5.22). After LH-20 purification of rice peptides, response of different fractions related with elongation of *Triticum aestivum* coleoptiles were again evaluated along with IAA as standard. IAA application at 10^{-5} (M) concentration enhanced coleoptile segments 160% higher than control. Unfortunately no significant changes in coleoptile growth were recorded after application of different fractions except in case of F_5, F_6 and F_{13}; where 20% to 40% excess elongation of coleoptile was observed from control with 1 ppm or lesser dose of peptide treatment (Table 5.6). Most significant coleoptile elongation by LH-20 fraction was found with 0.1 ppm F_6 peptide treatment, but this response was inhibited by application of higher doses of same peptide fraction (Table 5.6). Further bioassay guided purification with peptide fractions was not continued as highly significant bioactivity was not established by the treatment of different LH-20 fractions.

Table 5.7 Summary of bioactivity of ultrafiltered and column chromatographic fractions of different peptides isolated from seven days old *Oryza sativa* L. (rice) seedlings and their responses against different experiments. Sephadex LH-20 column (80 x 3 cm), volume – 565 ml approximately, eluted with 30% aqueous ethanol, and fractionated with 5 ml tube with pump speed – 30 ml/h. Tube number 1-26 or 130 ml is void volume approximately and any bioactivity, if found is rejected, and the rest 170 tubes were collected and screened for bioactivity

Fraction Tube Number:		**01-26**	**27-48**	**49-55**	**56-58**	**59-61**	**62-64**	**65-68**	**69-71**	**72-74**	**75-78**	**79-96**	**97-101**	**102-126**	**127-149**	**150-172**	**173-200**
Joined Fraction Number:		**VOID**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**
UV Absorbance:	214 nm	-	++++	++	+	+++	+++	++	+	+	-	+	-	-	+++	+++	-
	260 nm	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-
	280 nm	-	+	+	-	++	++	+	-	-	-	-	-	-	++	++	-
0.2% Ninhydrin response:		-	+++	+++	+	+++	+++	+++	++	++	+	+	-	-	+++	+++	-
α-Amylase elicitation:	Induction:	-N/A	-	-	+	++	++++	+++	+	-	-	-	-	-	-	-	-
	Inhibition:	-N/A	+	-	-	-	-	-	-	+	-	+	+	+	+	+	-
Control of stomatal guard cell:	Percentage of opening in dark	--N/A	+	+	-	+	++	++	+	+	++	-	-	+	-	+++	-
	Diameter of stomatal aperture	--N/A	-	-	-	+	+++	+	+	-	++	-	-	+	-	+++	-
	Percentage of closing:	No significant stomatal closure was observed in any fraction															
Growth of coleoptiles:	Elongation	--N/A	-	-	-	+	++	++	+	-	-	-	-	-	++	-	-
	Inhibition	--N/A	-	+	-	-	-	-	-	-	+	-	-	+	-	-	++
Seedling Growth Response:	Root:	Bioassay was not performed with rice peptides															
	Shoot:																
Retention of chlorophyll:		--N/A	+	-	+	-	+	+	+	-	-	-	-	-	+	+	-
Further purification with HPLC:		--N/A	-	-	-	-	6 peaks	-	-	-	-	-	-	-	-	5 peaks	-
Amino acid analysis of bioactive peptides:		--N/A	NOT DONE														
Sequencing of bioactive peptides:		--N/A	NOT DONE														

Relative quantum of peptides and response of different bioassays with Sephadex LH-20 purified peptide fractions of *Oryza sativa* were integrally represented in Table 5.7. First 26 tubes were considered as void and UV-absorbance were taken from fraction 1 to 15 at 214, 260 and 280 nm. UV-absorbance and 2% ninhydrin response of different peptide fractions were highly correlated and in both cases better response were revealed with F_1, F_4, F_5, F_6, F_{13} and F_{14} fractions (Table 5.7). When all bioassays were considered, amylase induction and stomatal opening response were attributed by rice peptides with fraction F_5 and F_{14} respectively in a potential manner. No significant stomatal closure and inhibition of amylase induction was established by these peptide fractions. Coleoptile elongation and chlorophyll retention activity was produced by peptide fractions in a diffused manner. As F_5 and F_{14} strongly represented amylase induction and stomatal opening response respectively, further bioassay-guided purification was continued with these fractions through HPLC. Ultimately 6 peaks and 5 peaks were obtained from HPLC fractions and their respective bioassays were also performed, but it was not possible for us to identify and sequence the final bioactive peptides.

In conclusion, it may be stated that the peptides isolated from rice seedlings mimic the action of hormones related with amylase induction and dark mediated stomatal opening. It was observed that the specific activity of peptides was decreased after certain stage of purification. During purification it was also noticed that more than one peptide fractions were responsible for regulating the same bioactivity. Further elucidation of bioactivity relationship of peptides with different signals will ultimately resolve the unknown issues of amylase induction and stomatal guard cell regulation during germination and post-germination seedling growth.

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